



PCT / CA 98 / 00992  
06 NOVEMBER 1998 (06.11.98)  
9/529925  
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Specification and Drawings, as originally filed with Application for Patent Serial No: 2,219,299, on October 24, 1997, by ELIAS GEORGES AND YING WANG, for "P-40, A New Member of the Multidrug Resistance Gene Family".

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November 6, 1998

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ABSTRACT OF THE DISCLOSURE

The present invention relates in general to multidrug resistance (MDR) in cells. In particular, the present invention relates to 5 the identification of a new member of the MDR gene family, P-40, as well as to the identification of P-40 related genes (homologs) as being further members of the MDR gene family. The present invention therefore relates to nucleic acid molecules encoding P-40 protein and P-40 protein homologs, to multidrug resistant cells containing these nucleic acid 10 molecules; to hybridomas containing antibodies to P-40 and P-40 homologs; to nucleic acid probes for the detection of these nucleic acid molecules; to a method of detection of such nucleic acid molecules or of the P-40 protein or P-40 homologs; to bioassays comprising the nucleic acid molecules encoding P-40 or P-40 homologs, P-40 protein or P-40 15 protein homologs, or antibodies of the present invention to diagnose, assess or prognose MDR in an animal; to therapeutic uses of the nucleic acid molecules of the present invention (i.e. antisense), protein or antibodies of the present invention; and to methods of preventing MDR in an animal.

**TITLE OF THE INVENTION****P-40, A NEW MEMBER OF THE MULTIDRUG  
RESISTANCE GENE FAMILY****5 FIELD OF THE INVENTION**

The present invention relates in general to multidrug resistance (MDR) in cells. In particular, the present invention relates to the identification of a new member of the MDR gene family, P-40, as well as to the identification of P-40 related genes (homologs) as being further members of the MDR gene family. The present invention therefore relates to nucleic acid molecules encoding P-40 protein and P-40 proteins homologs, to multidrug resistant cells containing these nucleic acid molecules; to hybridomas containing antibodies to P-40 and P-40 homologs; to nucleic acid probes for the detection of these nucleic acid molecules; to a method of detection of such nucleic acid molecules or of the P-40 protein or P-40 homologs; to bioassays comprising the nucleic acid molecules encoding P-40 or P-40 homologs, P-40 protein or P-40 protein homologs, or antibodies of the present invention to diagnose, assess or prognose MDR in an animal; to therapeutic uses of the nucleic acid molecules of the present invention (i.e. antisense), protein or antibodies of the present invention; and to methods of preventing MDR in an animal.

**BACKGROUND OF THE INVENTION**

25 The ability of malignant cells to develop resistance to multiple anticancer drugs is a major obstacle in the treatment of cancers [Ferguson et al., 1989, *Cancer Bulletin* 41(1): 7-13]. Studies using *in vitro*

model systems have led to the identification of several proteins which confer resistance to different classes of anticancer drugs [Pastan et al., 1987, *New England Journal of Medicine* 316(22): 1388-1393; Bradley et al., 1988, *Biochimica et Biophysica Acta* 948: 87-128]. The overexpression of 5 P-glycoprotein (P-gp) and the multidrug resistance associated protein (MRP) in cells selected with hydrophobic cytotoxic drugs (*Vinca alkaloids*, anthracyclines and epipodophyllotoxins) have been shown to confer a multidrug resistance phenotype [Gottesman et al., 1993, *Annual Review of Biochemistry* 62: 385-427; Endicott et al., 1989, *Annual Review of Biochemistry* 58: 137-171; Cole et al., 1996, *Cancer Treatment & Research*. 87: 39-62]. Both P-gp and MRP belong to a large family of ATP trafficking proteins 10 that are evolutionary conserved and mediate the transport of numerous ligand ranging from ions to large polypeptides [Higgins et al., 1992, *Annual Review of Cell Biology* 8: 67-113]. In tumor cell lines, P-gp and MRP reduce 15 the accumulation of drugs via an energy dependent drug efflux mechanism [Shapiro et al., 1994, *Journal of Biological Chemistry* 269(5): 3745-3754; Doige et al., 1992, *Biochimica et Biophysica Acta* 1109: 161-171].

P-gp and MRP expression has been detected in normal 20 tissues and is thought to mediate the transport of normal cell metabolites and xenobiotics [Cordon-Cardo et al., 1990, *Journal of Histochemistry and Cytochemistry* 38(9): 1277-1287; Bradley et al., 1990, *Journal of Cellular Physiology* 145: 398-408; Thorgeirsson et al., 1987, *Science* 236: 1120-1122; Thiebaut et al., 1987, *Proceedings of the National Academy of Sciences USA* 84: 7735-7738]. Consistent with these speculations, inactivation of both 25 alleles of P-gp from the mouse genome has led to the accumulation of drugs and natural products in many organs where P-gp is highly expressed [Schinkel et al., 1997, *Proceedings of the National Academy of Sciences of the United States of America*. 94(8): 4028-4033; Schinkel et al.,

1994, *Cell* 77: 491-502]. High levels of P-gp has been shown in 20 - 70% of tumors from different cancers [Tishler et al., 1992, *Journal of Neurosurgery* 76: 507-512; Abe et al., 1994, *Japanese Journal of Cancer Research* 85(5): 536-541; Baker et al., 1989, *Cancer and Metastasis Review* 8: 87-97; Belloni et al., 1989, *Cancer and Metastasis Review* 8: 353-389; Charpin et al., 1994, *Journal of the National Cancer Institute* 86(20): 1539-1545; Fojo et al., 1987, *Proceedings of the National Academy of Science USA* 84: 265-269; Henson et al., 1992, *Journal of Neuro-Oncology* 14: 37-43; Hijazi et al., 1994, *American Journal of Clinical Pathology* 102(1): 61-67; Mattern et al., 1994, *Anticancer Research* 14(2A): 417-419], and in some tumors (e.g. haemopoietic tumors and childhood malignancies) P-gp expression has been shown to predict clinical drug resistance and long term survival [Nooter et al., 1994, *Leukemia Research* 18(4): 233-243; Chan et al., 1995, *Hematology - Oncology Clinics of North America* 9(2): 275-318; Chan et al., 1991, *New England Journal of Medicine*, 325:1608-1614]. However, the lack of P-gp expression in other multidrug resistant tumors indicates other cellular changes that confer resistance to anticancer drugs [Lee et al., 1997, *Journal of Cellular Biochemistry*, 65(4): 513-526; Baggetto, *Bulletin du Cancer*, 84(4): 385-390; Linn et al., 1994, *Journal of Clinical Oncology* 12(4): 812-819; Lonn et al., 1994, *International Journal of Cancer* 58(1): 40-45; Sognier et al., 1994, *Biochemical Pharmacology* 48( 2): 391-401]. Some of the cellular changes identified in drug resistant cells include the overexpression of MRP [Zaman et al., 1994, *Proceedings of the National Academy of Sciences USA* 91(19): 8822-8826], alterations in glutathione-S-transferase activity or GSH levels [Tew, 1994, *Cancer Research* 54(15): 4313-4320], reduction in topoisomerase II levels or activity [Frelich et al., 1995, *J. Biological Chemistry* 270(15): 21429-21432], overexpression of LRP (the Lung Resistance Protein, a the component of human vaults) [36] and alteration

in functions or levels of proteins mediating apoptosis or programmed cell death [37, 38].

There thus remains a need to identify other cellular changes that confer drug resistance.

5 The overexpression of a 40 kDa protein (P-40) alone or together with P-gp or MRP in MDR cell lines [39] has been previously reported. However, further studies were required to demonstrate a direct role for P-40, if any, in drug metabolism and multidrug resistance (MDR). Indeed, it was disclosed that it was unknown and unclear whether P-40 10 could modulate a MDR phenotype directly or indirectly [39].

There thus remains a need to determine whether P-40 is indeed directly implicated in MDR. In the affirmative, there also remains a need to identify the molecular determinant of this MDR, in the form of a nucleic acid and for protein in order to open the way for the obtention 15 of diagnostic, therapeutic and research tools in the field of multidrug resistance.

The present invention seeks to meet these and other needs.

20 The present description refers to a number of documents, the content of which is herein incorporated by reference.

#### SUMMARY OF THE INVENTION

The invention concerns the demonstration that P-40 has a direct role in multidrug resistance.

25 Further, the invention relates to the identification of P-40 as a member of the MDR gene family and to the identification of

P-40/Annexin I related genes as members of this broadened MDR gene family.

In addition, the invention relates to the identification of Annexin II and IV as potential MDR determinants. Thus, the invention 5 relates to the identifications of Annexins (I to XI, also referred thereto herein P-40 and P-40 homologs) as potential members of the MDR gene family. Broadly therefore, the present invention also relates to the identification of Annexin-based MDR in cells.

The present invention further relates to the isolated 10 nucleic acid molecules encoding P-40 or fragment thereof and to the identification of P-40 as Annexin I.

The invention in addition relates to purified P-40 polypeptides, homologs thereof, or epitope binding portions thereof and the use thereof in multidrug resistance. The invention also provides a 15 specific detection method for P-40 nucleic acids encoding P-40 proteins or homologs thereof, polypeptides or fragments thereof in a sample.

In addition, the invention provides a recombinant nucleic acid molecule comprising P-40 (or homologs thereof) operationally linked to a promoter, efficient in initiating transcription thereof in a host cell as 20 well as to such a host cell.

As well, the invention provides a non-human organism containing the nucleic acid molecule mentioned above. Further, the invention provides an antisense P-40 or P-40-related (Annexin I-related) nucleic acid molecule.

25 The invention further provides an antibody having specific binding affinity towards P-40, P-40 homologs or an epitope-containing-region thereof. In one embodiment, the antibody is a

monoclonal. The invention also provides the hybridoma producing the monoclonal antibody.

5 The invention also seeks to provide a method for the detection of P-40 or P-40 homologs protein or portion thereof in a sample. In one embodiment, such a method is quantitative.

Furthermore, the invention seeks to provide a diagnostic kit comprising a first contained means containing the above-mentioned antibody, and second container means containing a conjugate comprising a binding partner of the monoclonal antibody and a label.

10 The invention seeks to provide diagnostic methods for human disease and particularly for cancer and the multidrug resistant of cancer cells. Preferably, a method for evaluating the predisposition of a cancer tumor to be and/or become multidrug resistance is provided herein.

15 The invention further seeks to provide therapeutic methods involving the P-40 nucleic acid homologs, variants or parts thereof, antisense thereof, P-40 protein, P-40 protein homologs or P-40 antibodies.

20 It shall be understood that in certain situations it might be beneficial to render a cell MDR by providing thereinto at least one nucleic acid encoding an Annexin (or at least one Annexin protein) so as to give this cell a growing advantage with respect to wild-type cells in certain growth conditions (i.e. presence of drug).

25 It is shown herein that P-40 is Annexin I. Annexin I is a member of a large family of Ca<sup>2+</sup> phospholipid binding proteins [for review see [40]], implicated in several cellular mechanisms including intracellular membrane vascular trafficking and exocytosis process

[41-43]. However, Annexin I has not been previously implicated in drug resistance or suggested to be implicated hereinto. The inventors are then the first to show the role of Annexin I in expression of drug resistance to anticancer drugs. Thus, the present invention is the first to show an

5 Annexin-based multidrug resistance pathway in cells.

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature

10 Commission.

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

15 As used herein, "isolated nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA and RNA molecules purified from their natural environment.

20 The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

25 The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

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The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are

selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement 5 amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

10 The nucleic acid (i.e. DNA or RNA) for practising the present invention may be obtained according to well known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. 15 In general, the oligonucleotide probes or primers are at least 12 nucleotides in length, preferably between 15 and 24 molecules, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hybridization thereof with its targeted sequence (see 20 below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

25 "Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals

referred above (Sambrook et al., 1989, and Ausubel et al., 1989) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with 5 a labeled probe in a solution containing 50% formamide, high salt ( 5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA ( i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the 10 desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known 15 methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al., 1989, *supra*).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and 20 α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

25 The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less prepared,

labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well known methods (Sambrook et al., 1989, *supra*). Non-limiting examples of labels include  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ , and  $^{35}\text{S}$ . Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma  $^{32}\text{P}$  ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labeled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and

ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide 5 which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See 10 generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification 15 (SDA), transcription-based amplification, the Q $\beta$  replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, Biotechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

20 Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat 25 stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is

complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products 5 using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization 10 following EtBr staining of the DNA following gel electrophoresis, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

15 Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, *Science* 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:392-396; and *ibid.*, 1992, *Nucleic Acids Res.* 20:1691-1696).

20 As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a specific polypeptide or protein. It will readily be recognized by the person of ordinary skill, that the 25 nucleic acid sequence of the present invention can be incorporated into

anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can 5 serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or 10 more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element 15 sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a 20 prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

As used herein, the designation "functional derivative" 25 denotes, in the context of a functional derivative of a sequence whether an nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially

similar to that of the original sequence. This functional derivative or equivalent may be a natural derivatives or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the 5 biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating 10 to a protein sequence, the substituting amino acid as chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the 15 present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can 20 be synthesized chemically or produced through recombinant DNA technology. all these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic 25 of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in Remington's Pharmaceutical

Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

5 As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position.

10 Spontaneous mutations and experimentally induced mutations exist. The result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

15 As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

20 While the property of a host cell to become MDR is demonstrated with human P-40/Annexin I other Annexins (II-XI), non-human Annexins, other biologically functional genes/cDNA-related to Annexins can also be used in the context of the present invention. For example, Mouse Annexin I could be used in some embodiments of the present invention as will be recognized by a person of ordinary skill.

25 Annexins are part of a gene family of multifunctional calcium - and phospholipid - binding protein (for a review see Raynald et al., 1994, Biochem., Biophys. Acta., 1197:45-62). They have been

described in many organisms from mammals, to molds and even plants, and their similar functional properties in  $\text{Ca}^{2+}$  and phospholipid are explainable by their common primary structure (Raynald et al., 1994, *supra*). Indeed, some of the Annexins are thought to have originated from 5 a common ancestor (Raynald et al., 1994, *supra*). Moreover, the family of Annexin genes shows very significant identity between human, rat, and mouse homologs (Raynald et al., 1994, *supra*). It will be clear to the person of ordinary skill that the present invention is not to be limited to 10 human Annexins as homologs having the biological function of Annexin-based MDR can be used within the context of the present invention.

Furthermore, since Annexins are found in diverse evolutionary distant organisms such as plants, yeasts, and parasites (Raynald et al., 1994, *supra*), the present invention has very broad implications. For example, the present invention opens the way to use 15 Annexins from diverse organisms, such as yeast (i.e. *Candida albicans*) and parasites as therapeutic targets. Antifungal drugs, for example, could be identified by using yeast Annexins as therapeutic targets.

The presence of Annexins in plants could find utility in the development of specific crop resistance, by for example, increasing 20 the expression level of at least one Annexin.

In accordance with yet another aspect of the present invention, there is provided a method of reducing Annexin-based MDR in a cell or animal, comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to the instant 25 invention.

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For pharmaceutical administration, the said polypeptide may be incorporated into preparations in either liquid or solid forms using

carriers and excipients conventionally employed in the pharmaceutical art, optionally in combination with further active ingredients. The preparation may, for example, be applied orally, parenterally, enterally or preferably topically. Preferred forms include, for example, solutions, 5 emulsions, gels, sprays, lotions, ointments, creams or powders.

One of ordinary skill can readily determine the amounts of Annexin-based MDR reducing agent to be administered. It is apparent that the dosage will be dependent on the particular treatment used. It should also be clear that the dosage should be chosen to display the 10 biological activity without causing adverse effects. It will be understood that age, sex, type of disease, of formulation and other variables known to the person of ordinary skill will affect determination of the dosage to be used.

The pharmaceutically acceptable carriers and excipients 15 are well known in the art. A representative textbook thereon is Remington's Pharmaceutical Sciences, 1980, 16th Ed., Mack Eds.

Advantageously the compositions may be formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredient. The total daily dose may, of course, be varied depending on 20 the subject treated and the particular use of the composition. This can obviously be adapted by the treating professional.

In general, techniques for preparing antibodies (including monoclonal metabolism and hybridomas) and for detecting 25 antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher,

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Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: *Antibody - A Laboratory Manual*, CSH Laboratories).

The present invention opens the way to the identification of agonists and antagonists of Annexins with respect to their role in MDR.

5 An assay for Annexin-based MDR activity in cells can be used to assess the effect of agents on Annexins function in drug resistance and therefore identify such agonists or antagonists. Non-limiting examples of such agents include nucleic acid molecules, peptides, antibodies, carbohydrates, or other pharmaceutical agents.

10 The present invention also provides polyclonal, monoclonal antibodies, or humanized versions thereof, chimeric antibodies and the like which inhibit or neutralize their respective Annexin targetted antigens *in vivo* and/or specific thereto.

Treatments comprise parenteral administration of 15 multiple or single doses of the above listed antibodies and derivatives thereof. The dosage will be varied by the practicing professional depending on the usual parameters such as pharmacodynamic characteristics the route of administration, recipient's characteristics, symptoms and/or disease thereof and the like. A daily dosage of active 20 ingredient can be for example about 0.1 to 100 mg/kg of body weight, ordinarily 0.5 - 50 and preferably 1-10 mg/kg per day, i.e. divided doses ranging from 1-6 times per day or alternatively in sustained release form.

The non-human animals of the present invention having 25 a transgenic interruption or alteration of the Annexin endogenous gene(s) (knock-out animal) and/or into the genome in which transgenes directing expression of Annexin(s) has been introduced include vertebrates such as rodents, non-human primates, amphibians, reptiles and the like. These

animals are prepared in accordance with known methods. The same applies to transgenic plants.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

5 Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

10 Figure 1A shows a restriction map of the gene encoding P-40. The map for the 1.4 kb fragment isolated from  $\lambda$ gt11 expression library with IPM96 monoclonal antibody. The restriction enzyme sites are indicated by the arrows. The solid bar indicates the coding sequence for P-40 or Annexin I. The thin lines to the left and right of the solid bar indicate the 5' and 3' non-coding regions. Figure 1B shows the nucleic acid sequence of P-40/Annexin I. Figure 1C shows the amino acid sequence of P-40/Annexin I. Figure 1D shows the nucleic acid sequence of both strands of P-40/Annexin I and hence shows examples of antisense nucleic acid molecules which can be used in accordance with 15 the present invention.

20 Figure 2 shows the *in vitro* expression of P-40. The *in vitro* expression of P-40 gene was performed using the T7 promoter directed transcription in TA PCRII cloning vector. Figure 2a shows *in vitro* transcribed and translated mixes containing PCRII vector only immunoprecipitated with IPM96 mAb or PCRII plus 1.4 kb insert immunoprecipitated with IPM96 mAb or an irrelevant IgG2b (lanes 2 and 25 3, respectively). Lanes 1-4 of figure 2b show the same samples as in lanes 1 and 2 but transferred to nitrocellulose membrane and probed with

a specific antibody to P-40 (IPM96 mAb) or an irrelevant IgG2b, respectively.

Figure 3 shows the analysis of protein and mRNA levels in drug sensitive and resistant cells. Total cell extract from drug sensitive (MCF7, SKOV3 and H69) and resistant human MDR cells (MCF7/AR, SKOV3/VLB<sup>1.0</sup> and H69/AR) were fractionated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with P-40 specific monoclonal antibody, IPM96. P-40 is seen in extracts from drug resistant cells (Lanes 4-6). Low levels of P-40 is detected in SKOV3 drug sensitive cells (lane 2) but not in MCF7 or H69 cells (1 and 3). For mRNA levels in the same cell lines, total RNAs were resolved on agarose gel and transferred to nylon membrane and probed with 32P labeled 1.4 kb fragment encoding P-40 or Annexin I and actin.

Figure 4 shows the post-translational modification of P-40 or Annexin I in MCF-7/AR cells. Cells were metabolically labeled with 35-methionine or 32P inorganic phosphate and radiolabeled protein were immunoprecipitated with an irrelevant IgG2b or IPM96 mAb (lanes 1 and 3 or 2 and 4, respectively).

Figure 5 shows the expression of Annexins I, II and IV in drug sensitive and resistant cells. Total cell extract proteins from drug sensitive (MCF-7, H69, SKOV3 and AuxB1) and resistant (MCF-7/Adr, H69/AR, SKOV3/VLB<sup>1.0</sup>, SKOV3/VLB0.06 and CH C5) or revertant (H69/PR) are resolved on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes are then probed with anti-Annexin I, II or IV (figures 5a, 5b and 5c, respectively).

Figure 6 shows the drug sensitivity assays for P-40 transient transfectants. MCF-7 cells transfected with pCDNA3 vector

without or with P-40 (Annexin I) gene are incubated in the absence and presence of increasing concentrations of Taxol (figure 6a) or doxorubicin (figure 6b). The level of P-40 (Annexin I) expression in MCF-7 transient transfectant cells was determined by Western blotting (figure 6c) and immuno fluorescence analysis (figure 6d).

5 Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

10

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

#### **MATERIALS AND METHODS**

15 **Cell culture and metabolic labelling**

Drug sensitive (MCF-7, H69, SKOV3 and HL60) and resistant (MCF-7/Adr, H69/Adr, SKOV3/VLB<sup>1.0</sup> and HL60/AR) cells were grown in the absence of antibiotics in  $\alpha$ -MEM or RPMI-1640 media supplemented with 5% to 15% fetal calf serum (Hyclon. Inc.) as 20 previously described [44-47]. Briefly, cells were grown at 37°C in humid atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were passaged when 70-80% confluent for adherent cells and 1X10<sup>6</sup> cell/ml for cells in suspension. Drug resistant cells were grown continuously with the appropriate concentrations of cytotoxic drugs 24 hours following subculturing. All cells 25 were examined for Mycoplasma contamination using the Mycoplasma PCR method (Stratagen Inc. San Diego, CA). For metabolic labeling of cells, MCF-7/Adr cells at 70-80% confluency were metabolically labeled

with [<sup>35</sup>S] methionine (100  $\mu$ Ci/ml; 1000 Ci/mmol; Amersham Life Sciences, Inc.) or Carrier free 32P inorganic phosphate (8 mCi/ml; Amersham Life Sciences, Inc.) for 3-4 hours at 37° C in methionine- or phosphate-free  $\alpha$ -MEM. Cells were lysed and the cell lysates were 5 immunoprecipitated with IgG<sub>2b</sub> or IPM96 mAb.

#### Screening an expression library with IPM96 monoclonal antibody

A 5' stretch cDNA expression library of HeLa cells constructed into  $\lambda$ gt11 vector was obtained from Clontech (Palo Alto, CA). 10 About 1X10<sup>6</sup> plaque forming units were plated using *Escherichia coli* Y1090 as host and screen with IPM96 monoclonal antibody. Briefly, plates containing phage plaques were incubated at 42°C for 4 hours and then overlayed with a dried nitrocellulose filter saturated in 10 mM X-gal. The plates were continuously incubated at 37°C for another 3 hours and 15 the filters were rinsed with TBST buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl, 0.05% Tween-20) and incubated in TBST containing 5% of skin milk for 30 minutes with gentle agitation. The nitrocellulose disks were incubated with TBST buffer containing 2  $\mu$ g/ml of Protein G purified IPM96 monoclonal antibody. The reactive plaques were detected with a 20 second goat anti-mouse IgG conjugated to horseradish peroxidase and visualized chemiluminescence using Amersham ECL kit. The immunoreactive plaques were verified by duplicate lifts and purified by subsequent rounds of screening using decreased plaque density. Immunoreactive plaques were eluted in ddH<sub>2</sub>O and utilized as template 25 for PCR directed by the 3' and 5' insert screening amplimer sequence of  $\lambda$ gt-11 and the fragment from PCR was cloned into PCR II vector (InVitrogen Inc.) following standard procedures [48].

### Nucleotide Sequencing and Computer Sequence Analysis

The cloned insert into the T/A PCR II vector was sequenced by the dideoxy chain termination method using M13 universal primer and sequence specific primers via the automated DNA sequencing 5 service at the Sheldon Biotech Centre at McGill University. Both strands of two different pick clones were completely sequenced. Computer analysis of the DNA and protein sequence was done using the Blast DNA search programs.

#### 10 Northern blot and DNA slot blot analyses

For Northern blot, total RNAs from drug sensitive and resistant cells were extracted with the Trazol solution (GibcoBRL, Gaithersburg, U.S.A). Approximately 10 µg of RNA from each cell line was run on a 1% formaldehyde-denatured agarose gel and transferred 15 to HybondsTM-N nylon membrane (Amersham, Oakvill, Ont.) by pressure blotting with 20X SSC (1X = 140 mM NaCl, 32 mM Sodium Citrate, pH 7.4). The blot was incubated in the presence of 32P labeled P-40 cDNA probe by nick translation or actin gene in 50% formamid, 2.5X Denhardt's solution, 25 mg/ml denatured Salmon sperm DNA, 1% SDS 20 and 1.25X SSPE overnight. The blot was washed in low stringency wash with 0.5X SSC for 30 minute at room temperature. A higher stringency wash was applied only if it was necessary. RNA integrity and equal loading was assessed in all cases by hybridization with actin or rRNA probes. Quantification of radioactive signals was carried out by scanning 25 the resultant autoradiography and analysis with NIH imaging software [49].

For slot blotting, genomic DNA from MCF-7 and MCF-7/Adr cells was prepared as described [48] and 10 µg DNA from each sample was denatured by 1 M NaOH. Following 10 minute boiling at 100°C. The denatured DNA was diluted serially loaded onto Nylon 5 membrane by using Manifold Slot Blot apparatus (Pharmacia Inc.). The blot was then hybridized with a 32P-labeled probe at 42°C in 50% formamide, 2.5XDenhardt's solution, 25 mg/ml denatured summon sperm DNA, 1% SDS and 1.25X SSPE overnight. The highest stringent wash of the blot was 0.5X SSC with 1% SDS at 65°C for 30 minutes.

10

#### **In vitro Translation and Immunoprecipitation**

*In vitro* transcription and translation reactions were carried out using a rabbit reticulocyte lysate (Promaga Corporation. Madison, WI) and 35S-methionine (Dupont/NEW, Mississauga, Ont.) 15 according to the manufacturer's protocol. Briefly, cDNA clone encoding full length P-40 was cloned into NotI and Xhol sites of a pCDNA3 expression vector containing T7 and SP6 promotor sequences (In Vitogene, Inc.). The pCDNA3 with and without P-40 insert were added to a coupled reticulocyte lysate transcription and translation system in the 20 presence of [35S]-methionine. Following a 2 hour incubation at 30°C, *in vitro* synthesized proteins were immunoprecipitated with IPM96 monoclonal antibody as previously described [50].

25

#### **DNA Transfection**

MCF-7 cells were transfected with the pCDNA3 vector or vector containing P-40 coding sequence using lipofectAMINE (Gibco, Burlington, Ontario, Canada) according to the manufacturer's procedure.

Briefly, 4 X 10<sup>5</sup> cells in a 60 mm plate with 5 ml of serum-free minimal essential medium were overlaid with 200  $\mu$ l of serum-free essential medium containing 5  $\mu$ g of supercoiled DNA mixed with 10  $\mu$ l of LipofectAMINE. After 5 hour incubation, the medium was replaced with

5 5 ml of minimal essential medium supplemented with 10% fetal calf serum (Hyclone Laboratories) and cells were further cultured at 37°C for 24 - 48 hours. For the transient transfection, cells were collected and the expression of P-40 was detected by western blot and immunofluorescence. For stable transfectants, G418 was added to the cells at

10 1 mg/ml and continuously selected for another two weeks. The individual transfectant clones were obtained by the limited dilution under the G418 treatment. A population exhibiting highly expressed P-40, designated BM-1, and a population of the cells transfected with pCDNA3 alone, designated AM-1 were expanded for further analysis by Western blot or

15 immunoprecipitation.

#### Immunofluorescence Staining of Cells

Cells were washed with PBS and smeared onto glass slides by brief centrifugation at 1,500 rpm. The cytopsins were air-dried and fixed for 10 minutes in ice-cold acetone. Cells were rinsed twice with PBS and incubated in 1% bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Slides were incubated with the IPM96 mAb (5  $\mu$ g/ml 1% BSA/PBS) for 30 minutes followed by three two minutes rinses with PBS. FITC-conjugated goat-anti-mouse IgG (1:50 dilution) was added to slides and allowed to incubate for 30 minutes. After several washes, slides were mounted in PBS containing 50% glycerol and examined with a Nikon UFX-DX fluorescent microscope fitted with a 60X

oil immersion objective. Photographs were taken with Kodak Tri-X pan film (400 ASA) at 800X magnification.

#### **Drug Sensitivity Assay**

5                   Transient transfectants were harvested 48 hours later and aliquoted at 7.5X10<sup>3</sup> cells per well of a 96-well plate. Drugs were added to cells 24 hours later and incubated for another 48 hours at 37°C. The assay was developed by adding 50 µl (5 mg/ml) of an MTT dye and allowed to incubate for four hours at 37°C as previously described [51].

10

#### **SDS-PAGE and Western Blotting**

15                   Total cell lysates cell cultures or from transcription/translation reaction mixture were mixed with equal volumes of sample buffer containing SDS and the denatured proteins were resolved on 10% polyacrylamide gels according to the method of Laemmeli [52]. For acrylamide gels containing 35S-methionine labeled proteins, gels were fixed in 50% methanol/water and soaked in Amplify™ (Amersham, Oakville, Ont.) for 30 minutes prior to drying and exposing to Kodak film at -70°C. For Western blot analysis, proteins were 20 transferred to nitrocellulose membrane for 1 hour at 50 volts according to the method of Towbin et al., [53]. The nitrocellulose membrane was blocked with 5% skin milk/PBS and incubated overnight at 4°C with 1 µg/ml of IPM96 monoclonal antibody. The immunoreactive proteins were 25 detected with horseradish peroxidase conjugated goat anti-mouse antibody and visualized by chemoluminence using Amersham ECL kit (Amersham, Oakville, Ont.).

## RESULTS

### Isolation and characterization of P-40 cDNA clones

In a previous report [39], we had demonstrated the overexpression of a 40 kDa protein (P-40) in several MDR cell lines alone or together with P-gp and MRP. In this study, we have used the monoclonal antibody IPM96 which binds specifically to P-40 to screen a cDNA expression library made from HeLa cells. A total of 50,000 plaques were screened and several positive plaques were identified following the initial screening. Of the latter positive plaques two positive clones were obtained after sequential plaque purification and both inserts were isolated by PCR (see Material and Methods). Both positive clones encoded for a 1.6 kb fragments that were subsequently cloned into a T/A PCRII vector. Sequence analysis of both clones showed an open reading frame of 346 amino acids which is consistent with the molecular mass of the protein (38.7 kDa versus 40 kDa) (figure 1A). Comparison of P-40 nucleotide and amino acid sequences (figures 1B and 1C) to other sequences in the DNA data bank, using the DNA search programs TBLast, showed it to be identical to Annexin I sequence [54].

To confirm the identify of the isolated cDNA, a 1.4 kb encoding the full length of P-40 (Annexin I) was expressed *in vitro* using T7 promotor directed transcription-translation reticulocyte lysate with 35S-methionine. Figure 2a shows the immuno- precipitation of proteins with IPM96 mAb from an *in vitro* expression reactions containing vector only (lane 1) or vector plus 1.4 kb insert (lane 2). As a control for the IPM96 antibody, an irrelevant IgG2b was used to immunoprecipitate proteins from a reaction mix containing vector plus the 1.4 kb insert (figure 2a, lane 3). The results of figure 2a show a 35S-methionine

labeled 40 kDa protein immunoprecipitated with IPM96 mAb but not with an irrelevant IgG2b (lanes 2 and 3, respectively). Figure 2b, also shows Western blotting of the proteins identical to those in lanes 1 and 2 of figure 2a, but probed with IPM96 mAb (lanes 1 and 2) or an irrelevant IgG2b (lanes 3 and 4). Taken together, these results confirm the identity of the 1.4 kb fragment as the gene encoding P-40 or Annexin I.

To determine if the amino acid sequence of P-40 (or Annexin I) cloned from HeLa cells is similar or different from that found in MCF-7/AR cells, Annexin I was cloned from MCF-7/AR cells by reverse 10 PCR using 5' and 3' primers encoding Annexin I. Sequence analysis of Annexin I clones from MCF-7/AR cells revealed no differences from that isolated from HeLa cells cDNA expression library (data not shown).

#### **Northern blot analyses**

15 In an earlier study [39], the levels of P-40 was compared between drug sensitive and resistant cell lines. Figure 3a shows a Western blot analysis of total cell lysates from drug sensitive (MCF-7, SKOV3 and H69) and their resistant (MCF-7/AR, SKOV3/VLB<sup>1.0</sup> and H69/AR) cells probed with IPM96 mAb. The results of the latter Western 20 blot analysis shows clearly the increase in P-40 expression in resistant cells relative to the parental drug sensitive cells. The SKOV3 cells show lower levels of P-40 than the resistant SKOV3/VLB<sup>1.0</sup>. However, it is interesting that the SKOV3 cell line was derived from a patient with ovarian tumor that was considered to be clinically resistant Cis-platinum 25 and adriamycin [55]. To determine if the increase in P-40 or Annexin I protein expression in the above MDR cells is due to an increase in mRNA levels, Northern blot analysis were performed with total RNA extracted

from drug sensitive (MCF-7, SKOV3 and H69) and resistant (MCF-7/AR, SKOV3/VLB<sup>1.0</sup> and H69/AR) cells and blotted membrane probed with a 32P labeled 1.4 kb fragment. The results in figure 3b shows the 1.8 kb mRNA band in resistant MCF-7/AR and H69/AR but not in the parental drug sensitive cell lines, MCF-7 and H69/AR. The level of 1.8 kb mRNA hybridizing band was 4-fold higher drug resistant SKOV/VLB<sup>1.0</sup> cells relative to drug sensitive SKOV-3 cells (figure 3b). Taken together, the Northern blot demonstrate clearly that the observed increase in P-40 or Annexin I cells is due to an increase in mRNA of P-40. Furthermore, the Northern blot results are consistent with the Western blot data, especially those relating to the protein and mRNA levels of P40 or Annexin I in SKOV3 versus SKOV3/VLB<sup>1.0</sup> cells (figure 3).

To determine if the above increase in mRNA levels in MDR cells relative to drug sensitive cells is at the transcriptional level or is due to gene amplification, genomic DNA was isolated the above cells lines and analyzed quantitatively using slot blot. The results (data not shown) show no gene amplification for P-40 or Annexin I between drug sensitive and resistant cells.

**20 Post-translational modification of P-40 or Annexin I in resistant cells**

Annexin I is a phosphoprotein phosphorylated at serine and tyrosine amino acids [56, 57]. Further, it has been shown that phosphorylation of Annexin I at its N-terminal domain decreases its affinity for negatively charged phospholipids or membrane vesicle aggregation [56]. Given the above results, it was of interest to examine the post-translational modification of P-40 or Annexin I in MDR cells. The results in figure 4 show immuno-precipitation of P-40 or Annexin I with an

irrelevant IgG2b or IPM96 mAb from MCF-7/AR cells that have been metabolically labeled with 35-methionine (lanes 1 and 2) or 32P inorganic phosphate (lanes 3 and 4). Interestingly, P-40 or Annexin I was not phosphorylated in MCF-7/AR cells. Similarly, it was not possible to 5 demonstrate basal level of P-40 or Annexin I phosphorylation in the other MDR cells (data not shown).

#### **Overexpression of Annexins II and IV in MDR cells**

To determine if other member of the annexin family are 10 similarly overexpressed in MDR cells relative to the parental drug sensitive cells, total cell proteins from drug sensitive (MCF-7, H69 and SKOV3) and resistant (MCF-7/AR, H69/AR and SKOV3/VLB<sup>10</sup>) cells were separated by SDS-PAGE and transferred to nitrocellulose membrane. Figure 5 shows the results of the Western blots probed with 15 anti-Annexin I, II and IV. The results of the Western blot probed with anti-Annexin I show similar results to the Western blot in figure 3a probed with IPM96, confirming the antigen specificity of IPM96 mAb towards to Annexin I. Furthermore, the expression of Annexin II and IV is also increased in MDR cells relative to the parental cell lines, however to a 20 lesser extend as that of Annexin I. For example, both Annexin II and IV are expressed at lower levels in drug sensitive cells compared to MDR cells (figure 5). Of considerable interest is the levels of Annexin I, II and IV in a revertant cell line (H69/PR) derived from H69/AR cells that are less resistant to doxorubicin (figure 5). cDNA transfections (transient and 25 stable) of Annexins II-XI, as described below will be carried out to verify the drug resistance of Annexin II-XI-transfected cells.

**cDNA transfection and drug sensitivity analyses**

In an attempt to investigate the role of P-40 or Annexin I overexpression in drug resistance, the full length gene encoding P-40 or Annexin I was cloned into a mammalian expression vector, pCDNA3 and transfected 5 into MCF-7 cells. MCF-7 cells transfected transiently with pCDNA3 alone or with P-40 gene are cultured for 3 days prior to analysis. The results in figure 6a show a Western blotting of total cell proteins from MCF-7 cells transfected with pCDNA3 vector only or pCDNA3 vector plus P-40 or Annexin I gene (lanes 2 and 3). The results in lane 3 of figure 6a show a 10 40 kDa protein in MCF-7 cells transfected with pCDNA3 vector plus P-40 or Annexin I. To determine more quantitatively, the level of P-40 transfection following a three day culturing, MCF-7 cells transfected with pCDNA3 plus P-40 or Annexin I full length cDNA were analyzed by 15 indirect immunofluorescence with IPM96 mAb. The results in figure 6b show the relative number (<5%) of MCF-7 cells that overexpress P-40 or Annexin I. The efficiency of the transient transfection to ~5% of the cells was confirmed following transfection with pCDNA3 containing a beta-gal 20 gene (data not shown).

Having established the expression of P-40 or Annexin I 25 in MCF-7 cells, it was of interest to examine the effect of P-40 or Annexin I overexpression (albeit <5%) on the sensitivity of MCF-7 cells to anticancer drugs. The results in figure 6c and 6d show MCF-7 cells transfected with vector only and with vector plus P-40 incubated with increasing concentrations of Taxol or adriamycin, respectively. Surprisingly, overexpression of P-40 or Annexin I in MCF-7 cells 25 decreases their sensitivity to Taxol and adriamycin. Although it is expected, whether higher levels of P-40 or Annexin I expression will lead

to a larger decrease in the sensitivity of transfected cells to anticancer drugs will have to be formerly tested using for example stable cell lines expressing PCDNA3 alone or with P-40 gene.

5 The transient transfected cells were incubated with a chelator (EGTA) or a calcium channel blocker (Verapamil). P-40-protein was shown to be released by the membrane and the EGTA or Verapamil-treated cells were also shown to have reduced drug resistance to taxol or adriamycin. This results suggest that small molecules find utility in the context of the present invention.

10

## DISCUSSION

15 In this study we have used the monoclonal antibody IPM96, previously shown to detect a 40 kDa protein in MDR cells to screen a  $\lambda$ gt11 expression library. Two positive  $\lambda$ gt11 clones were identified and their cDNA insert was isolated by PCR and cloned into a TA PCRII vector. Analysis of the nucleotide and amino acid sequence of the 1.4 kb cDNA insert revealed an open reading frame of 346 amino acids that are identical to Annexin I [54]. *In vitro* expression of IPM96 positive cDNA clone using a transcription-translation retic, lysate followed by immuno precipitation and Western blot analyses of the expressed 40 kDa protein confirmed the identify of the 1.4 kb fragment as P-40. In addition, Northern blot analysis using total RNA from drug sensitive and resistant cells confirmed the overexpression of Annexin I or P-40 mRNA in MDR cells relative to their parental drug sensitive cells.

20

25 Besides the similarities in the P-40 and Annexin I molecule masses on SDS-PAGE and the cross-reaction of IPM96 with Annexin I expressed *in vitro*, the identity of the P-40 as Annexin I is

consistent with our earlier observations where P-40 was shown to be found both in the membrane and soluble fractions [39]. Furthermore, extraction of membrane associated P-40 was resistant to high salt and EDTA and suggests the possibility that some of P-40 may be an integral membrane protein. Interestingly, a similar conclusion was independently suggested for Annexin I in an earlier study . The latter possibility is likely given that annexins I, V, VI and VII possess ion channel activity [58, 59]. Also consistent with our assignment of P-40 as Annexin I, is the fact that the 35 kDa proteolytic product which has been previously demonstrated to represent the head domain of Annexin I, is highly sensitive to proteolysis [56].

Annexin I is a member of a large family of calcium dependent membrane binding proteins that are sometimes referred to as lipocortin, calpeptins, endonexins (for review [40]). Annexins share a similar core domain with four or eight conserved 70 amino acid repeats and an amino terminal domain that varies in length and sequence between the different members of the annexin family. Although the physiological function(s) of annexins is not clear, they have been implicated in calcium-regulated exocytosis [60, 61]. Annexin I has also been shown to mediate the calcium-dependent fusion of liposomes with isolated neutrophil plasma membranes [62]. In intact cells, annexins are generally phosphorylated in response to varieties of stimuli. Annexin I is phosphorylated by EGF receptor-kinase at tyrosine residues in found in the N-terminal head domain [63] and by protein kinases C and A [57]. Interestingly, phosphorylation of Annexin I at the amino terminal domain by protein kinase C inhibits its ability to aggregate chromaffin granules [64]. In adrenal chromaffin cells, Annexin I was shown to be rapidly

phosphorylated upon stimulation of cells to secrete normal cell metabolites [65]. Taken together, our finding that Annexin I is not phosphorylated in MDR cells is consistent with its increased capacity to cause aggregation of membrane vesicles.

5           In this report we show, for the first time, a direct role of Annexin I overexpression in tumor cells resistance to anticancer drugs. Using transient transfections of MCF-7 tumor cells, we showed that transfection of P-40 or Annexin I cDNA confers resistance to Taxol and adriamycin. Although the levels of resistance towards the latter drugs are  
10 only 1.2 to 2.0-fold more than control cells (transfected with vector alone), the results are consistent and are not surprising in light of the percentage (<5%) of transfected cells (figure 6a and 6b). Furthermore, similar transfection studies of P-gp or MRP cDNA have also shown much lower levels of drug resistant in transfectant cells when compared to selected  
15 MDR cells that overexpress similar amounts of these proteins [33, 66-68]. Whether Annexin I confers lower levels of drug resistance as compared to P-gp or MRP drug efflux mechanisms remains to be determined. In any event, unlike *in vitro* tumor selected cell lines, low levels of drug resistance conferred by P-40 or Annexin I are likely to be clinically  
20 relevant. Work is in progress to isolate and characterize the drug resistant profile of stable transfectants of Annexin I.

Although, our data thus far shows a direct correlation between P-40 or Annexin I overexpression and resistance to anticancer drugs, it is no clear how is P-40 mediated this phenotype. However, given  
25 the role of annexins in promoting aggregation of membrane vesicles through calcium phospholipid binding, it may be speculated that Annexin I confers a drug resistance phenotype by promoting the aggregation of

drug filled membrane vesicle or exocytosis of such drug filled vesicles [69]. Increased membrane vacuolization has been observed in many MDR selected cell lines [70, 71]. Furthermore, P-gp and MRP have been observed in endosomal membranes in *in vitro* selected MDR cell lines

5 [72-74] In the latter possibility, Annexin I or other members of the annexin family (which are also overexpressed in MDR cells; figure 5) could function together with P-gp or MRP to cause the aggregation and possibly exocytosis of drug filled vesicles.

Of importance, the analysis of Annexin I RNA levels in

10 mouse and hamster was shown to also positively correlated with MDR.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

## REFERENCES

1. Ferguson et al., 1989, *Cancer Bulletin* 41(1): 7-13
2. Pastan et al., 1987, *New England Journal of Medicine* 316(22): 1388-  
5 1393
3. Bradley et al., 1988, *Biochimica et Biophysica Acta* 948: 87-128
4. Gottesman et al., 1993, *Annual Review of Biochemistry* 62: 385-427
5. Endicott et al., 1989, *Annual Review of Biochemistry* 58: 137-171
6. Cole et al., 1996, *Cancer Treatment & Research*. 87: 39-62
- 10 7. Higgins et al., 1992, *Annual Review of Cell Biology* 8: 67-113
8. Shapiro et al., 1994, *Journal of Biological Chemistry* 269(5): 3745-3754
9. Doige et al., 1992, *Biochimica et Biophysica Acta* 1109: 161-171
10. Cordon-Cardo et al., 1990, *Journal of Histochemistry and Cytochemistry* 38(9): 1277-1287
- 15 11. Bradley et al., 1990, *Journal of Cellular Physiology* 145: 398-408
12. Thorgeirsson et al., 1987, *Science* 236: 1120-1122
13. Thiebaut et al., 1987, *Proceedings of the National Academy of Sciences USA* 84: 7735-7738
14. Schinkel et al., 1997, *Proceedings of the National Academy of Sciences of the United States of America*. 94(8): 4028-4033
- 20 20 15. Schinkel et al., 1994, *Cell* 77: 491-502
16. Tishler et al., 1992, *Journal of Neurosurgery* 76: 507-512
17. Abe et al., 1994, *Japanese Journal of Cancer Research* 85(5): 536-541
18. Baker et al., 1989, ? : 87-97
- 25 19. Belloni et al., 1989, *Cancer and Metastasis Review* 8: 353-389
20. Charpin et al., 1994, *Journal of the National Cancer Institute* 86(20): 1539-1545
21. Fojo et al., 1987, *Proceedings of the National Academy of Science USA* 84: 265-269

22. Henson et al., 1992, *Journal of Neuro-Oncology* 14: 37-43
23. Hijazi et al., 1994, *American Journal of Clinical Pathology* 102(1): 61-67
24. Mattern et al., 1994, *Anticancer Research* 14(2A): 417-419
25. Nooter et al., 1994, *Leukemia Research* 18(4): 233-243
- 5 26. Chan et al., 1995, *Hematology - Oncology Clinics of North America* 9(2): 275-318
27. Chan et al., 1991, *New England Journal of Medicine*, 325:1608-1614
28. Lee et al., 1997, *Journal of Cellular Biochemistry*, 65(4): 513-526
29. Baggetto, *Bulletin du Cancer*, 84(4): 385-390
- 10 30. Linn et al., 1994, *Journal of Clinical Oncology* 12(4): 812-819
31. Lonn et al., 1994, *International Journal of Cancer* 58(1): 40-45
32. Sognier et al., 1994, *Biochemical Pharmacology* 48(2): 391-401
33. Zaman et al., 1994, *Proceedings of the National Academy of Sciences USA* 91(19): 8822-8826
- 15 34. Tew, 1994, *Cancer Research* 54(15): 4313-4320
35. Frelich et al., 1995, *J. Biological Chemistry* 270(15): 21429-21432
36. Scheffer et al., 1995, *Nature Medicine* 1(16): 578-578
37. Lowe et al., 1993, *Cell* 74(6): 957-967
38. Lowe et al., 1994, *Science* 266(5186): 807-810
- 20 39. Wang et al., 1997, *Biochemical & Biophysical Research Communications*, 236(2): 483-488
40. Raynal et al., 1994, *Biochimica et Biophysica Acta*, 1197: 63-93
41. Creutz, 1992, *Science*, 258: 924-931
42. Lin et al., 1992, *Cell* 70: 283-291
- 25 43. Creutz et al., 1992, *Journal of Cell Science*, 103: 1177-1192
44. Mirski et al., 1987, *Cancer Research* 47: 2594-2598
45. McGrath et al., 1987, *Biochemical & Biophysical Research Communications*, 145(3): 1171-1176
46. Bradley et al., 1989, *Cancer Research* 49: 2790-2796

47. Batist et al., 1986, *Journal of Biological Chemistry*. 261(33): 15544-15549
48. Sambrook et al., 1989, *Molecular cloning: A laboratory Manual*. Cold Spring Harber Laboratory Press, Cold Spring Harbor, N.Y.
- 5 49. Wayne R, 1992, *NIH Imaging Software*
50. Georges et al., 1991, *Journal of Cellular Physiology* 148: 479-484
51. Pouliot et al., 1997, *Biochemical Pharmacology* 53(1): 17-25
52. Laemmli et al., 1970, *Nature* 227(259): 680-685
53. Towbin et al., 1979, *Proceedings of the National Academy of Sciences of the United States of America* 76(9): 4350-4354
- 10 54. Wallner et al., 1986, *Nature* 320: 77-81
55. Fogh et al., 1975, Plenum Press, New York : 155-159
56. Wang et al., 1994, *BIOCHEMISTRY* 33: 276-282
57. Varticovski et al., 1988, *BIOCHEMISTRY* 27: 3682-3690
- 15 58. Pollard et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 2974-2978
59. Rojas et al., 1990, *Journal of Biological Chemistry* 265(24239-24245)
60. Drust et al., 1988, *Nature* 331: 88-91
61. Creutz et al., 1987, *Journal of Biological Chemistry* 262: 1860-1865
62. Meers et al., 1986, *Nature* 321(81-84.)
- 20 64. Wang et al., 1986, *Journal of Biological Chemistry* 261(6548-6553.)
66. Cole et al., 1994, *Cancer Research* 54(22): 5902-5910
67. Ueda et al., 1987, *Proceedings of the National Academy of Sciences USA* 84: 3004-3008
68. Gros et al., 1986, *Nature* 323: 728-731
- 25 69. Chauffert et al., *Cancer Research* 46: 825-830
70. Beck, 1987, *Biochemical Pharmacology* 36(18): 2879-2887
71. Sehested et al., 1987, *British Journal of Cancer* 56: 747-751
72. Abbaszadegan et al., 1997, *Cancer Research* 56(23): 5435-5442

73. Slapak et al., 1992, *Journal of Biological Chemistry*. 267(15): 10638-10644
74. Klohs et al., 1988, *Molecular Pharmacology* 34: 180-185

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**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid molecule encoding an Annexin family member, a member of the multidrug resistance (MDR) gene family for assessing or modulating MDR in a cell.  
5
2. The isolated nucleic acid molecule of claim 1, wherein said cell is selected from an animal cell, mammalian cell, a human cell, a parasitic cell and a fungal cell.  
10
3. The isolated nucleic acid molecule of claim 1, comprising a nucleic acid sequence which is at least 90% identical to a sequence selected from the group consisting of a nucleotide sequence encoding an Annexin polypeptide selected from Annexin I to Annexin XI, or a nucleotide sequence complementary thereto.  
15
4. The isolated nucleic acid molecule of claim 1, 2 or 3, wherein said Annexin is Annexin I and said Annexin polypeptide is the Annexin I polypeptide which comprises the complete amino acid sequence set forth in Figure 1C.  
20
5. The isolated nucleic acid molecule of claim 1 or 2, wherein said Annexin is Annexin I and said nucleotide sequence comprises the nucleotide sequence as set forth in Figure 1B.  
25

6. A method of detecting and/or assessing Annexin-based MDR in a sample comprising:

- 5        a) contacting said sample with an isolated nucleic acid molecule consisting of 10 to 50 nucleotides specifically hybridizing to RNA and/or DNA encoding an Annexin, wherein said nucleic acid molecule is or is complementary to a nucleotide sequence consisting of at least 10 consecutive nucleotides from the nucleic acid sequence of one of Annexin I to XI, under hybridization conditions; and
- 10        b) detecting the presence of said molecule bound to Annexin nucleic acid.

7. A kit for detecting and/or quantifying an Annexin nucleic acid in a sample comprising at least one container means having disposed therein an isolated nucleic acid molecule consisting of 10 to 50 nucleotides specifically hybridizing to RNA and/or DNA encoding an Annexin, wherein said nucleic acid molecule is or is complementary to a nucleotide sequence consisting of at least 10 consecutive nucleotides from the nucleic acid sequence of one of Annexin I to XI.

8. A recombinant vector for modulating and/or inhibiting and/or increasing Annexin-based MDR in a cell comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter element.

9. The recombinant vector of claim 8, wherein Annexin is Annexin I.

10. The recombinant vector of claim 9, being  
5 PCDNA3/P-40.

11. A cell that contains the recombinant vector of claim  
8.

10 12. A method of identifying compounds that affect Annexin-based MDR, said method comprising:

15 a) incubating the cell of claim 11 in the presence of a potential Annexin-based MDR-affecting compound in the presence and absence of a drug; and  
b) assessing the effect of said compound on the resistance to said drug.

13. The method of claim 12, wherein said compound is selected from the group consisting of a nucleic acid molecule, a dominant  
20 negative mutant of an Annexin, a mutant Annexin, an antibody to Annexin, a peptide, and a small molecule.

14. The method of claim 13, wherein said compound is an Annexin I antisense molecule.

15. A method of reducing Annexin-based MDR in a cell comprising: administering thereto a therapeutically effective amount of a compound selected from the group consisting of a nucleic acid molecule, a dominant negative mutant of an Annexin, a mutant Annexin, an antibody to Annexin, a peptide, and a small molecule.

5 16. The method of claim 15, wherein said compound is an Annexin I antisense molecule.

10 17. The method of claim 15, wherein said compound is a calcium chelator or a calcium channel blocker.

15 18. A pharmaceutical composition for reducing MDR in a cell comprising an Annexin-based MDR affecting compound together with a suitable pharmaceutically acceptable carrier.

19. A method of diagnosing the presence or predisposition of Annexin-based MDR in a patient comprising:

20 a) taking a sample from said patient;  
b) determining the amount of Annexin protein and/or RNA in said sample;  
c) diagnosing the presence or predisposition of Annexin-based MDR in said patient, wherein an increased amount of said Annexin protein and/or RNA in said sample as compared to a control sample

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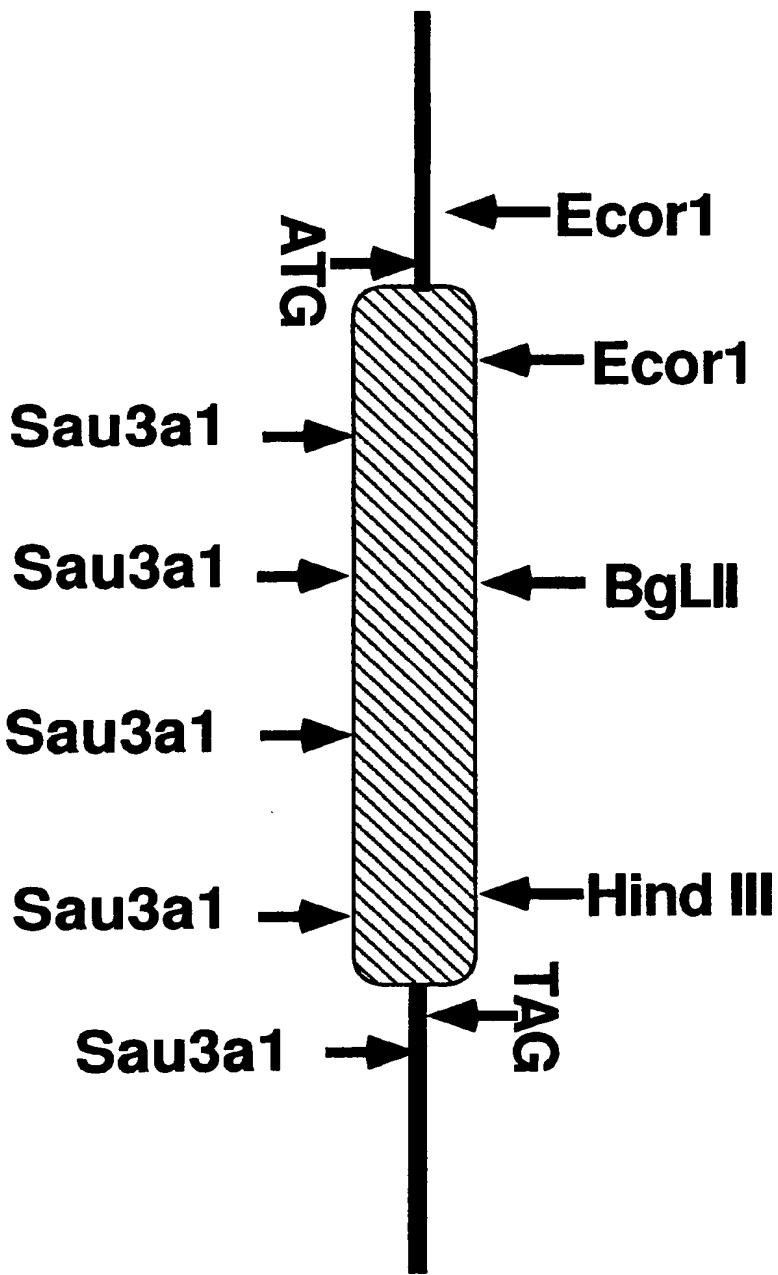
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indicates the presence or predisposition towards Annexin-based MDR.

20. A method of diagnosing the presence or  
5 predisposition of Annexin-based MDR in a pathogen comprising:  
a) taking a sample from said pathogen;  
b) determining the amount of Annexin protein and/or  
RNA in said sample;  
c) diagnosing the presence or predisposition of  
10 Annexin-based MDR in said pathogen, wherein an  
increased amount of said Annexin protein and/or RNA  
in said sample as compared to a control sample  
indicates the presence or predisposition towards  
Annexin-based MDR.

# Restriction map of the gene encoding for P-40

Figure 1A



## ORIGIN

11

Figure 1.c

= "MAMVSEFLKQAWFIENEEQEYVQTVKSSKGPGSAVSPYPTFNP  
SSDVAALHKAIMVKGVDEATIIDLTKRNNAQRQQIKAAYLQETGKPLDETLKKALTG  
HLEEVVLALLKTPAQFDAELRAAMKGLGTDEDTLIELASRTNKEIRDINRVYREEL  
KRDLAKDITSDTSGDFRNALLSLAKGDRSEDFGVNEDLADSARALYEAGERRKGTDV  
NVFNTILTRSYPLRQVFQKYTKYSKHDMMNKVLDLELKGDIEKCLTAIVKCATSKPA  
FFAEKLHQAMKGVGTRHKALIRIMVSRSIDMNDIKAFYQKMYGISLCQAILDETKGD  
YEKILVALCGGN"

10 20 30 40 50 60  
 GT AGC GAC CGC TCA GCT GCA CCG GGC CGC GTC GAT TTT TTT GTC TGG TGA CTC CAA CTC GTC  
 CA TCG CTG GCC CGC ACT CGA CCT CCG CCG CGC CGC CTC AAA AAA CAG AAC ACT GCA GTC  
 <A V P A S S A A A D I K K D Q S T M N  
 <L S R R E A P I R P R T S K K T K H R K I  
 <Y R G A S L Q F E R R G R R R N R Q R T V D N R  
 <R P A L S W N S R P R R P F C L V T S P Y  
 S D R R S A G I R G R V D F P V L R H P I  
 A T G A Q L E F A A A S I F L S C D V I L F  
 70 80 90 100 110 120 130  
 TTC AGC TAC ATA GAC ATC TTT CTC ATG TPA CTA GAA CAA CTT GTC TAG GGT TTT ATG GTT TGG GGA  
 AAG TCG ATG TAT CTG TAG AAA GAG TAC ATG GAT CTT GTC GAA CAT ATC CCA AAA TAC CAA ACC CCT  
 <E A V Y V D K E H F L K Y L T K H N P S  
 <K L H S M K R M N S S C S T Y P K I T Q P P  
 <S C L C R E T V V L V V Q I P N P K P P  
 F Q L H R H L S H V T R T T C I G F Y G L G  
 F S Y I D I F L M L L E Q L V G P M V W G  
 S A T T S F S C Y N N L Y R V L W F G E  
 140 150 160 170 180 190 200  
 AAA CAT TTT TAA AAA ATG GAC TTA TCT CTA TTA TAC AGA GTC ATA ATA TAA AAA TGA TTT AAA GGC  
 TTT GTC AAA ATT TTT TAC CTG AAT AGA GAT ATG TCT CAA TAT TAT ATT TTT ACT AAA TTT CCG  
 <F M K b P H V R V S N Y Y L F S K P A  
 <P C K F P I S K D R N Y L T I I Y F H N L P  
 <V N K P F P S I E T I I C L L I F I I L S  
 K T F L K N G L I S I I Q S Y N I K M I R  
 K H F K M D L S L L Y R V I I K F K G  
 N I F K K W T Y L Y Y T E L Y K N D L K A  
 210 220 230 240 250 260  
 TAT ATT TTT CAG CAT GTC GAT AGC TAC ACT GTC ATC CTG TTG AAG AAA CTT TCC TAT TTA AGC TTA  
 ATA TAA AAA GTC GTC CAT CCA TCG ATG TGA CAT TAG GAC AAC TTC TTT GAA AGG ATA AAT TCG AAT  
 <I N K L M Y T A V S Y D Q Q L F K G I A  
 <I K C T P L V T I R N F F S E R L K  
 <Y K E A H L Y S C O L G T S S V X R N L S I  
 L Y F S A C R L H C N P V E E T F L F K L  
 Y I F Q H V G S Y T V I L L K X L S Y L S L  
 I F F S H V A T L S C R N F P I A Y  
 270 280 290 300 310 320 330  
 TAG GAT GAA AAT ATA TAA TTA AAG TCT TCT GAT CAT AGC TTG AGA CCA TCA AGG GAA TGT TTA GTT  
 ATC CTA CTT TTA TAT ATT AAT TTC AGA AGA AGA GTC AAC TCG AAC TCT GGT AGT TCC CTT ACA AAT CAA  
 <L I P I Y L L R R I M A Q S W P F T N  
 <Y S S F I Y N F D E S L K L G D L S H K T  
 <P H F Y I I L T K Q D Y S S V M L P I N L K  
 I G K Y I I K V F S L E T I K G M F S  
 D E N I L K S S D H S L R P S R E C L V  
 R M K I Y N S L L I I A D H Q G N V F  
 340 350 360 370 380 390  
 TCC TCC ACA AAG AGC CAC CAG GAT TTT CTC ATA ATC TCC TTT GGT TTC ATC CAG GAT GGC TTG GCA  
 AGG AGG TGT TTC TCG GTG GTC CTA AAA GAG TAT TAG AGG AAA CCA AGG TAG GTC CTC CGG AAC CGT  
 <G C C L A V L I K E Y D G K T E D L I A Q C  
 <E E V F P L W W S K R M I E K P K M W S P K A  
 <R W L S G G P N E L R R Q N G P H S P L  
 F L H K E P P G F S H N L L W F H P G W L G  
 S S T K S H Q D F L I I S P G F I Q D G L A  
 P P Q R A T R I F S S P L V S S R M A W Q  
 400 410 420 430 440 450 460  
 AAG GGA GAT ACC ATA CAT CTT CTG ATA GAA TGC TTT GAT ATC ATT CAT GTC AAT TTC AGA ACG GGA  
 TTC CCT CTA TGG TAT GTC GAA GAC TAT CTT AGC AAA CTA TAG TAA GTC CAG TTA AAG TCT TGC CCT  
 <L S I G Y M R Q Y P A K I D N M D I E S R S  
 <P P S V M C R R I S H K S I M T L K L V P  
 <P L Y W V D E S L I S Q Y E H N F P P F  
 K G R Y B T S S D R M L Y H S C Q F Q N G  
 K G D T I H L L I E C F D I I H V N F R T G  
 R E I P Y I F N A L I S F M S I S E R  
 470 480 490 500 510 520 530 540  
 AAC CAT AAT CCT GAT CAA TGC CTT ATT GCG AGT TCC AAC ACC TTT CAT GGC TTG ATG AAG CTT CTC TGC AAA GAA AGC T  
 TTG GTC TTA GGA CTA GTT AGC GAA TAA CGC TCA AGG TGG AAA GTC CGG AAC TAC TTC GAA GAG AGC TTT CTT TCG A  
 <V M I R I L A K N R T G V G K H A Q H L K E A F F A  
 <P W L G S H R I A L E L V K P K I F S R Q L S L  
 <G Y D Q D I G Q S N W C R E H S S S A E R C L F  
 K P S S M P Y C E F Q H L S W L D E A S L Q R K L  
 N H N P D Q C L I A S S N T F H G L M K L C K E S X  
 T I I L I N A L L R V P T P F M A S F S A K K A

FIGURE  
1.D

**Figure 2a**

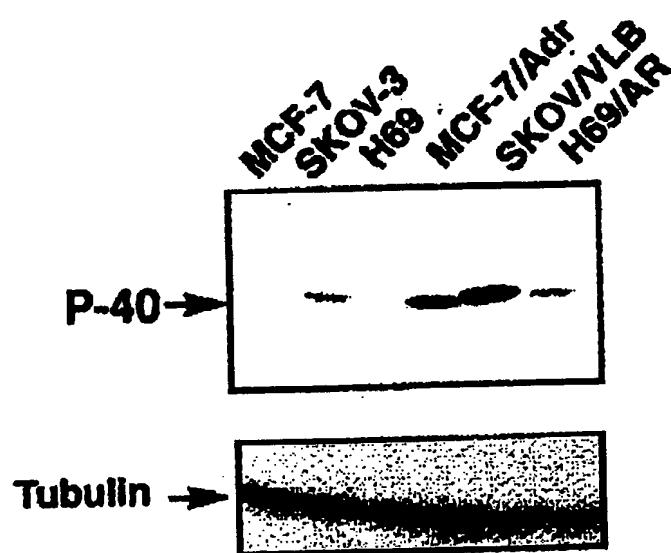


Figure 2b.

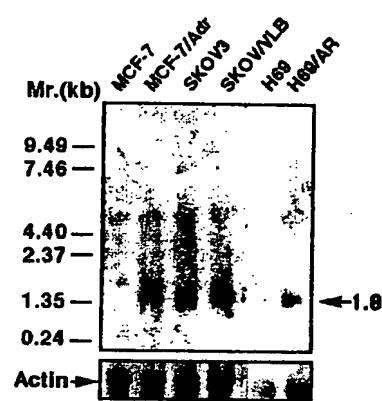


Figure 3

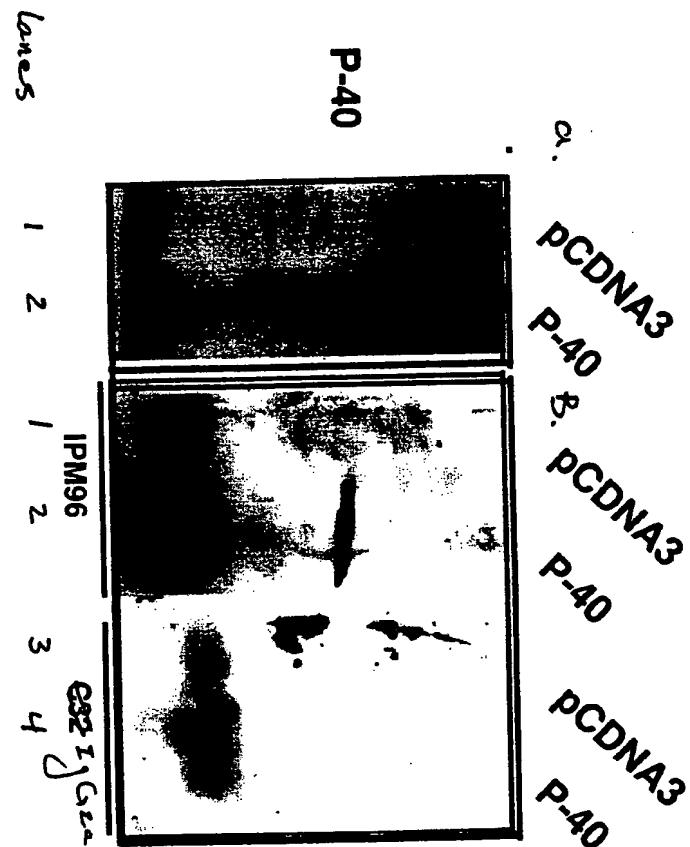


Figure 4.

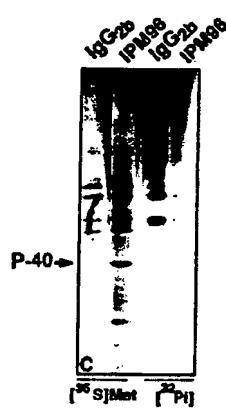
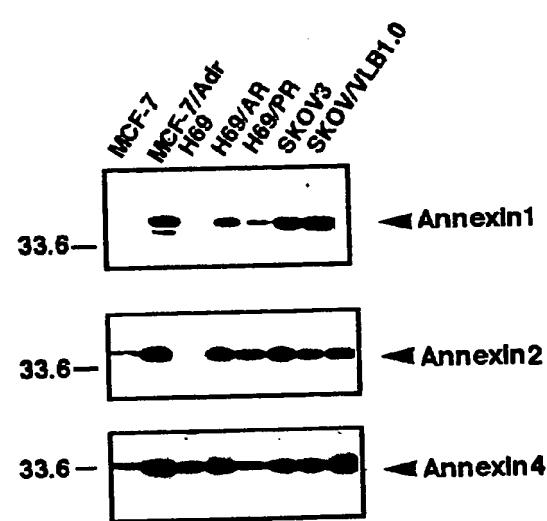


Figure 5.



Westernblot Analysis Of Cells transfected With The  
Gene Encoding For P-40

Figure 6 a.

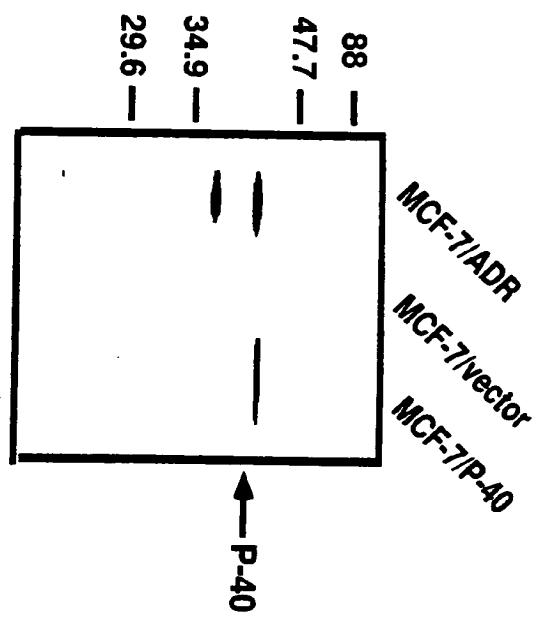


Figure 6 b.

Slide follow.  
Color picture.

Figure 6 b.

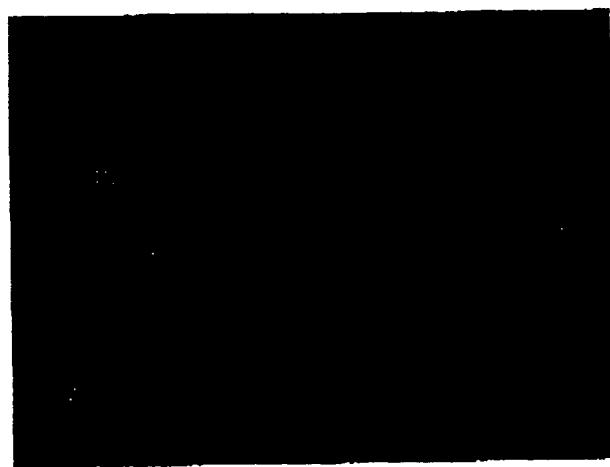


Figure 6c.

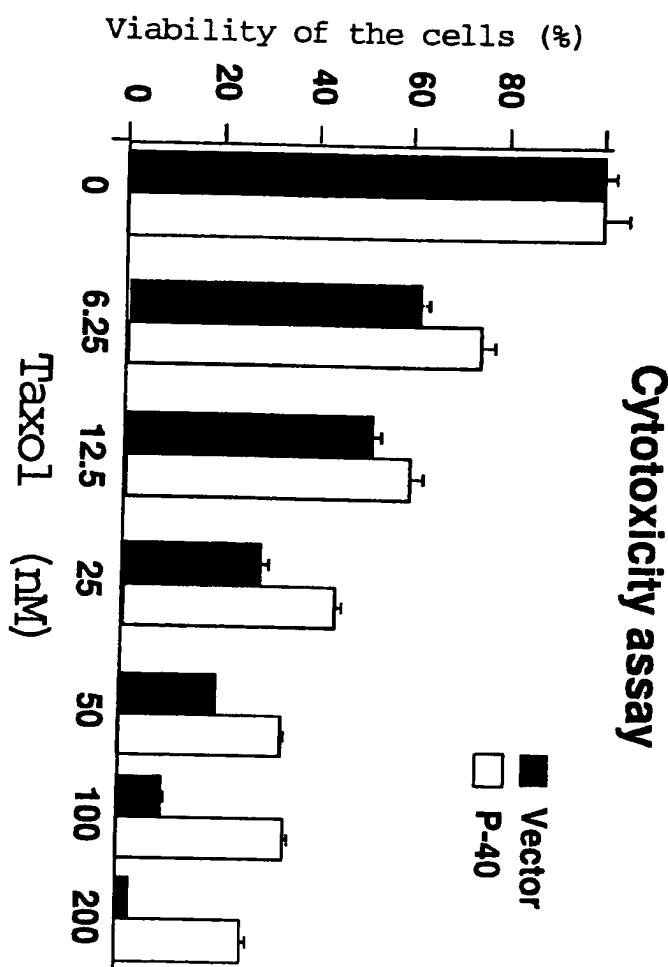


Figure 6D. Cytotoxicity assay

